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Enhancing the separation of phosphorylated proteins in gel electrophoresis with dinuclear bispyridylmethylamine–tyrosine–acrylamide complexes

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ABSTRACT

Bispyridylmethylamine–tyrosine–acrylamide ligands were prepared from protected tyrosine and dipyridylmethylamine by a twofold Mannich reaction and converted into acryl amides. Their manganese complexes were used as gel additives to increase the specific phosphate affinity in SDS–PAGE protein separations. The modified gel showed a distinct mobility shift of phosphorylated α-casein in comparison to the dephosphorylated protein.

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1. Introduction

Post-translational modification of proteins by phosphorylation is fundamental in all living organisms for the regulation of biological processes, such as signal transduction, apoptosis, proliferation, differentiation and metabolism [1]. This modification is very abundant with up to one third of all cellular proteins being phosphorylated. In eukaryotic cells, the hydroxyl groups of serine, threonine and tyrosine are phosphorylated [2]. Incorrect protein phosphorylation can cause severe disorders including cancer and neuropathogenesis. Methods for determining the phosphorylation status of proteins are important for a better understanding of the molecular origin of diseases and biological and pathological processes. Historically, phosphorylation detection methods rely on either radioisotopes or phosphoamino acid-selective antibodies. More recently, specific dyes for phosphorylation detection in arrays [3], biosensors [4], synthetic fluorophores [5] for kinase activity and artificial phosphoprotein sensors based on dinuclear Zn_2^{4+} metal complexes (Scheme 1) have been described [6–8].

Koike et al. combined the specific reversible coordination of metal complexes to phosphorylated protein surfaces with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE), the standard biochemical technique for protein separation and analysis. Derivative **3** of their hydroxyl-bis-DPA (dipicolylamine) ligand bearing a polymerizable acryl amide moiety (called Phos-tag™ by Koike et al., Scheme 1) was immobilized within the polyacrylamide gel and subsequently treated with zinc(II) or magnesium(II) ions [9]. In aqueous solution, the di-zinc(II) phos-tag complex strongly binds to phenyl phosphate ($K_d = 2.5 \times 10^{-8}$ mol/L) under physiological conditions, whereas at pH > 9 the binding ability decreases. The dinuclear manganese(II) phos-tag complex captures $R{-}OPO_3^{2-}$ anions preferentially, such as phosphoserine and phosphortyrosine at alkaline pH of approx. 9 [10,11]. These modified gels retain phosphorylated proteins longer than the corresponding non-phosphorylated proteins in SDS–PAGE gel electrophoresis which allows for detection of their mobility shift [12–15].

However, the preparation of compound **3** requires a multistep protocol [16]. We report here the facile preparation of ligand **4** from tyrosine, formation of its zinc(II) and manganese(II) complexes and their use as a mobility shift additive for the detection of phosphorylated proteins in SDS–PAGE gel electrophoresis (see Schemes 2 and 3).

2. Results and discussion

The twofold Mannich reaction of Boc-Tyr-OMe **5-Boc**, dpa **6** and paraformaldehyde leads to ligand **7-Boc** according to a known literature procedure in 51% yield [17,18]. The Boc protecting group is removed by treatment with HCl saturated ether and the crude product reacted with acrylchloride to give the target acrylamide-pendant ligand **4** in an overall good yield.

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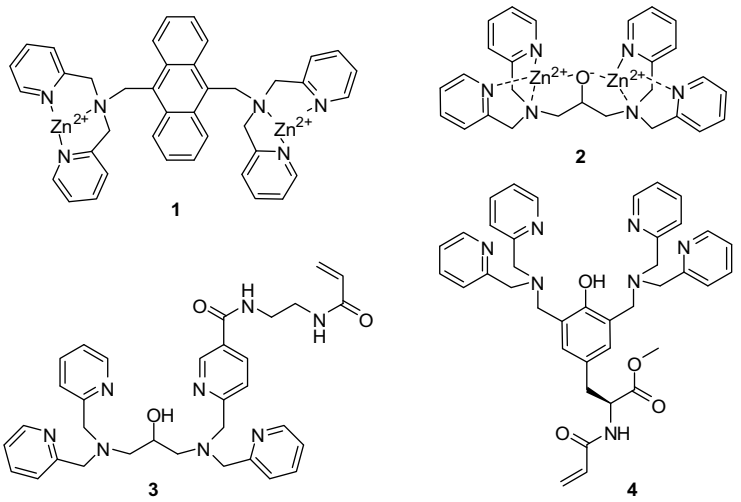
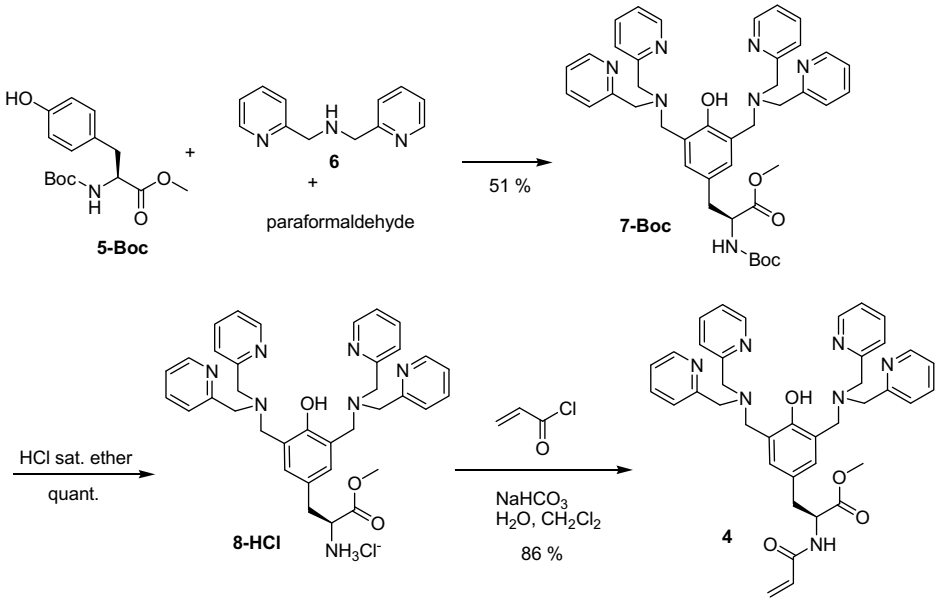


Scheme 1. Hamachi's (top left) and Koike's (top right) phosphate-binding dinuclear metal complexes **1** and **2**; polymerizable additives for the detection of phosphorylated proteins by mobility shift in gel electrophoresis: Phos-tag™ **3** (bottom left), a tyrosine-based ligand **4** (bottom right).



Scheme 2. Synthesis of acrylamide-pendant ligand **4** in three steps from protected tyrosine.

For some applications a modification of the acryl amide position may be desirable. Using **5-Cbz** as starting material, the protected analogue **7-Cbz** is obtained from twofold Mannich reactions in the same yield. Saponification of the methyl ester with LiOH gave compound **9**, which was reacted with mono Boc-protected diethylenediamine under standard peptide coupling conditions to give amide **11-Boc**. Boc-deprotection and reaction with acrylic acid chloride gave ligand **12** in an overall good yield. The ligand is converted into the di-manganese(II) or di-zinc(II) complex by treatment with the respective chloride salt in aqueous methanol solution.

The ability of the compounds to specifically retain phosphorylated proteins was investigated by SDS–PAGE, which was conducted according to Laemmli's method [19]. Either acrylamide-pendant ligands **4** or **12** with 2 equiv. of $MnCl_2$ or the metal complexes **12-Mn** or **12-Zn** were added in different amounts to the acrylamide mixture before polymerization of the separating gel. Only the manganese(II) complex led to retardation of phosphorylated proteins. No significant difference in retardation was observed if the ligand was used in the polymerization and its complex was formed subsequently or the metal complex was directly used in gel preparation. The phospho-protein α-casein from bovine milk was analyzed along with trypsinogen serving as a non-phosphorylated control. Both proteins were incubated either in the absence or presence of λ-protein phosphatase and then subjected to SDS–PAGE. Polyacrylamide gels were prepared either with or without the addition of 100 μM of **12-Mn**. Fig. 1 shows that the retardation of the phosphorylated α-casein is more pronounced in the presence of **12-Mn** in the gel, while untreated or phosphatase-treated samples of trypsinogen comigrated under both conditions.

To investigate if the observed mobility change was caused by masking the phosphate charges or by altered interactions of the phosphorylated protein with the polymeric gel structure, complex **12-Mn** or other coordination compounds (see Supplementary material for data) were added to the protein sample to be analyzed.

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